Institute for Reference Materials and Measurements

ERM European Reference Materials

CERTIFICATION REPORT

Certification of Plasmid DNA containing 98140 Maize DNA Fragments

Certified Reference Material ERM®-AD427

EUR 24722 EN – 2011

JRC EUROPEAN COMMISSION

The mission of the JRC-IRMM is to promote a common and reliable European measurement system in support of EU policies.

European Commission
Joint Research Centre
Institute for Reference Materials and Measurements

Contact information
Reference materials sales
Retieseweg 111
B-2440 Geel, Belgium
E-mail: jrc-irmm-rm-sales@ec.europa.eu
Tel.: +32 (0)14 571 705
Fax: +32 (0)14 590 406

http://irmm.jrc.ec.europa.eu/
http://www.jrc.ec.europa.eu/

JRC 63151

EUR 24722 EN
ISBN 978-92-79-19277-7
ISSN 1018-5593
doi:10.2787/38563

Luxembourg: Publications Office of the European Union

Printed in Belgium

CERTIFICATION REPORT

Certification of Plasmid DNA containing 98140 Maize DNA Fragments

Certified Reference Material ERM®-AD427

M. Caprioara-Buda, P. Corbisier, D. Gancberg, S. Broeders, S. Trapmann, S. Mazoua, G. Matayron, H. Emons

European Commission, Joint Research Centre
Institute for Reference Materials and Measurements (IRMM), Geel (BE)

Disclaimer

Certain commercial equipment, instruments, and materials are identified in this report to specify adequately the experimental procedure. In no case does such identification imply recommendation or endorsement by the European Commission, nor does it imply that the material or equipment is necessarily the best available for the purpose.

ABSTRACT

This report describes the preparation, characterisation, stability and suitability of the certified reference material (CRM) ERM®-AD427, which contains a plasmid (pIRMM-0090) carrying a defined 2'-deoxyribonucleic acid (DNA) fragment specific for a genetic modification present in *Zea mays* L. line 98140, as well as a defined DNA fragment specific for the *Zea mays* taxon.

The CRM was processed in 2008 and certified in 2011 by the European Commission, Joint Research Centre, Institute for Reference Materials and Measurements (IRMM), Geel, BE. The CRM is available in plastic tubes containing 500 µL of the plasmid DNA in 1 mmol/L Tris, 0.01 mmol/L EDTA, pH 8.0 buffer (TE) and 1 ng/µL ColE1 plasmid used as background DNA. Each tube contains approximately 10^9 copies of the ERM-AD427 plasmid, which corresponds approximately to 3.3 ng of DNA.

The plasmid contains a 80 bp fragment specific for the maize 98140 and referred as the 5' insert-to-plant junction. Additionally, the plasmid contains a 79 bp fragment of the maize endogenous *high mobility group* (*hmg*) gene.

The certified values are the numbers of cloned DNA fragments for the 98140 and *hmg* PCR targets per plasmid.

The recommended minimum sample intake is 50 µL to perform a dilution series. A minimum sample intake of 5 µL was used per real-time Polymerase Chain Reaction (PCR) assay.

	Number of DNA fragments per plasmid	
	Certified value [2]	**Uncertainty** [3]
Fragment of 5' insert-to-plant junction DNA / plasmid pIRMM-0090[1]	1	negligible
Fragment of *hmg* DNA / plasmid pIRMM-0090[1]	1	negligible

[1] The sequence identity has been confirmed by dye terminator cycle sequencing of the 5' insert-to-plant junction and *hmg* fragments present in *Zea mays* DP-Ø9814Ø-6, on plasmid pIRMM-0090. The estimated error probability of the sequence identification of each fragment is lower than 0.00003 %.

[2] The certified value is traceable to the International System of Units (SI).

[3] The uncertainty related to the sequencing method is a standard uncertainty. It is estimated by a type B evaluation based on the information provided in Section 8.1 of this certification report.

The intended use of this CRM is for the calibration of the event-specific method for the quantification of the 98140 event validated by the European Union Reference Laboratory for GM Food and Feed (EURL-GMFF), as described in the "Protocol for event-specific quantification of DP-Ø9814Ø-6 maize", accessible on the homepage of EURL-GMFF (http://gmo-crl.jrc.c.europa.eu/statusofdoss.htm)

GLOSSARY

35S	35S promoter, derived from cauliflower mosaic virus
6-FAM	6-carboxylfluorescein dye
α	error probability
A	absorbance
ANOVA	analysis of variance
BLASTN	Basic Local Alignment Search Tool for Nucleotides
bp	base pair
cp	copy number
CRM	Certified Reference Material
Ct value	cycle threshold, number of PCR cycles to pass a set threshold
DNA	2'-deoxyribonucleic acid
ds	double-stranded
EDTA	ethylenediaminetetraacetic acid
ENGL	European Network of GMO Laboratories
ERM®	trademark of European Reference Materials
EURL-GMFF	European Union Reference Laboratory for GM Food and Feed, formerly referred to as Community Reference Laboratory for Genetically Modified Food and Feed (CRL-GMFF)
ε	PCR efficiency
gDNA, gDNA$_\ell$, gDNA$_s$	genomic DNA, genomic DNA extracted from leaves, genomic DNA extracted from seeds
GM	genetically modified
GMO	genetically modified organism
hmg	*high mobility group* gene (taxon-specific gene)
IncQ	plasmid incompatibility group Q
IQR	interquartile range
IRMM	Institute for Reference Materials and Measurements
LB	Luria-Bertani
M	molar mass
n	number of replicates
N	number of tubes analysed
N_A	Avogadro constant
N_d	number of data sets
N_{ds}	number of data sub-sets
NCBI	National Centre for Biotechnology Information
NIH	National Institutes of Health
oriV	plasmid origin of replication
p	probability
PCR	polymerase chain reaction
pDNA	plasmid DNA
R^2	coefficient of determination
rel	relative (subscript used to describe relatively expressed values)
RNA	ribonucleic acid
rpm	rotations per minute
S	size of a DNA fragment expressed in number of bp
s	standard deviation
SI	International System of Units
TBE	Tris-Borate-EDTA
TE	Tris-EDTA
Tris	tris(hydroxymethyl)aminoethane
U	expanded uncertainty
UNG	uracil N-glycosylase
UV	ultraviolet
$\bar{x}$	mean
$\bar{x}_i$	mean obtained for data set i
x_t	DNA copy number ratio measured at the test temperature t
z	estimated error probability per base in DNA sequencing

TABLE OF CONTENTS

1 INTRODUCTION

Legislation in the European Union demands the labelling of food products consisting of or containing "more than 0.9 % genetically modified organisms" (GMOs), provided the GMO has been placed on the market in accordance with Community legislation [1]. This enforces the necessity, on the one hand to develop and validate reliable quantitative measurement methods, and on the other hand to develop and produce reference materials to calibrate and control the correct application of detection methods. This threshold (0.9 %) is commonly understood as a mass fraction. In October 2004, the European Commission recommended expressing the content of GM food and feed as the percentage of GM-DNA copy numbers in relation to target taxon-specific DNA copy numbers calculated in terms of haploid genomes [2].

Calibrants are required to determine the ratio of the number of copies of transgenic and taxon-specific genes. Here we describe the development of a new pure DNA calibrant containing a DNA sequence specific for the 98140 event and a DNA fragment containing a *Zea mays* taxon-specific sequence. As for any plasmid harbouring unique sequences, the ratio between the sequences is equal to 1.

The identity of both cloned sequences has been confirmed by the dye terminator cycle sequencing method. The nucleotide sequence of the 5' insert-to-plant junction cloned into the plasmid calibrant was identical to the confidential nucleotide sequence provided by Pioneer Hi-Bred International, Inc. (Johnston, IA, US).

Likewise, the nucleotide sequence of the *hmg* fragment cloned into the plasmid calibrant was identical to the nucleotide sequence referred to as AJ131373.1 in the National Institutes of Health (NIH, Bethesda, MD, US) genetic sequence database (GenBank®), and corresponds to the *Zea mays hmg* gene present in *Zea mays* species [3].

The maize 98140 event is registered in the Organisation for Economic Co-operation and Development Unique Identification Registry (available via http://bch.cbd.int/database/lmo-registry/), as specified in the Regulation (EC) No 65/2004 of 14/01/2004 [4] establishing a system for the development and assignment of unique identifiers for GMOs. The maize 98140 event received the unique identifier DP-Ø9814Ø-6.

2 DESIGN OF THE PROJECT AND CERTIFICATION PROCEDURE

The major objective of the project was the production of a plasmid DNA reference material for the calibration of real-time PCR quantification methods.

The material is intended to be used as calibrant for the quantification of the 98140 event expressed as the ratio between the number of copies of the 98140 event and the number of copies of a taxon-specific *Zea mays hmg* gene. The plasmid calibrant is certified for containing one single DNA fragment of these two genes per plasmid. The cloned DNA fragments are identical to the sequences published in the National Institute of Health (NIH, Bethesda, MD, US) genetic sequence database (GenBank®).

The relative number of copies of the 98140 event per haploid genome maize that are present in a DNA extract can be calculated by application of the EURL-GMFF validated quantification method, using the ERM-AD427 calibrant.

3 PARTICIPANTS

Processing, stability study and value assignment

European Commission, Joint Research Centre, Institute for Reference Materials and Measurements (IRMM), Geel, BE* (BELAC 268-TEST)

Characterisation

Eurofins Medigenomix GmbH, Martinsried, DE
European Commission, Joint Research Centre, Institute for Reference Materials and Measurements (IRMM), Geel, BE

Suitability study

Agencia Española de Seguridad Alimentaria - Centro Nacional de Alimentación, Madrid, ES* (ENAC 178/LE397)
Danish Plant Directorate, Laboratory for Diagnoses in Plants, Food and Feed, Lyngby, DK* (DANAK, 330)
Danmarks Tekniske Universitet, DTU - New Technical University of Denmark, Fødevareinstituttet, Søborg, DK* (DANAK, 350)
Ente Nazionale Delle Sementi Elette ENSE, Tavazzano, IT
Eurofins Genescan GmbH, Freiburg, DE* (DACH, DAC-PL-0526-07-03)
European Commission, Joint Research Centre, Institute for Health and Customer Protection (IHCP), Ispra, IT* (DACH, DAC-PL-0459-06-00)
European Commission, Joint Research Centre, Institute for Reference Materials and Measurements (IRMM), Geel, BE* (BELAC, 268-TEST)
Groupe d'études et de contrôle des variétés et des semences GEVES (BioGEVES), Surgeres, FR* (COFRAC, 1-1540)
Hainaut Vigilance Sanitaire, Institut Provincial d'Information et d'Analyses Sanitaires, Mons, BE* (BELAC, 068-test)
Institut für Hygiene und Umwelt, Behörde für Umwelt und Gesundheit, Hamburg, DE* (DACH, DAC-PL-0137-01-10)
Instituut voor Landbouw- en Visserijonderzoek (ILVO), Dept. of Animal Product Quality and Transformation Technology, Merelbeke, BE* (BELAC, 033-TEST)
Korea Research Institute of Standards and Science (KRISS) - Organic and Bio Analysis Group, Daejeon, KR
Lebensmittel- und Veterinäruntersuchungsamt des Landes Schleswig-Holstein (LVUA), Landeslabor Schleswig-Holstein, Neumünster, DE* (AKS, AKS-P-10101-EU)
Livsmedelsverket - National Food Administration, Biology Division, Uppsala, SE* (SWEDAC, 1457)
Nacionalinis maisto ir veterinarijos rizikos vertinimo institutas (NMVRVI), Vilnius, LT* (DAP, DAP-PL-3328.99)
Nacionalni Inštitut za Biologijo - National Institute of Biology (NIB), Ljubljana, SI* (Slovenska akreditacija, LP-028)
Nederlandse Organisatie voor toegepast-natuurwetenschappelijk Onderzoek (TNO), TNO quality of life - Food & Biotechnology Innovations - GMO foods, Zeist, NL* (Dutch Accreditation Council RvA, L027)
Nestlé Research Center, Lausanne, CH* (SAS, STS 188)
Tullilaboratorio - Finnish Customs Laboratory, Espoo, FI* (FINAS, T006)
Umweltbundesamt Wien, Wien, AT* (Federal Ministry of Economics and Labour, 200)

* Laboratory holds accreditation ISO/IEC 17025 for DNA based GM measurements (accreditation body and registration number are mentioned).

4 DESCRIPTION AND PROCESSING OF THE MATERIAL

4.1 DESCRIPTION OF THE STARTING MATERIAL

4.1.1 Cloning of the transgenic target

A 80 bp fragment containing part of the maize chromosome and part of the 98140 transgenic sequence (5' insert-to-plant junction sequence) was amplified by the Platinum Taq DNA polymerase (Invitrogen, Carlsbad, CA, US) using genomic DNA extracted from seedlings raised from seeds of maize 98140 as template. The PCR product was purified using Qiagen PCR purification kit (QIAGEN Benelux B.V., Venlo, NL) and ligated into the ligation-ready pCR®2.1 vector (Invitrogen, Carlsbad, CA, US); *Escherichia coli* (*E. coli*) Top10 cells were transformed with the ligation products. The transformed cells were selected on LB plates supplemented with 50 µg/mL kanamycin and 100 µg/mL ampicillin, and identified by qualitative PCR screening using primers flanking the border regions of the cloned fragment. The correct insertion was confirmed by restriction enzyme digestion with *Eco*RI (resulting in 2 bands of 98 bp and 3913 bp) and *Hind*III/*Xba*I (resulting in 2 bands of 192 bp and 3819 bp) on plasmid DNA isolated from single colonies. One single clone bearing the plasmid named pIRMM-0089 was selected.

4.1.2 Cloning of the endogenous target

A 351 bp fragment of the endogenous *high mobility group* (*hmg*) gene from maize was amplified by the Taq polymerase (Invitrogen, Carlsbad, CA, US) using genomic DNA extracted from MON 810 *Zea mays* as template. The amplicon was cloned into a pCR®2.1 vector (Invitrogen, Carlsbad, CA, US) to yield the plasmid pIRMM-0034. This plasmid was digested with *Eco*RI to release the fragment specific for the *hmg* gene. Subsequently, this fragment was ligated into the *Eco*RI restricted pUC18 vector (pIRMM-0012) using the rapid DNA ligation kit (Roche, Mannheim, DE) to yield the plasmid pIRMM-0067; *E. coli* Top10 cells were then transformed with the ligation products. The transformed cells were selected on 100 µg/mL ampicillin-containing LB plates and identified by qualitative PCR screening using primers flanking the border regions of the cloned fragment. The correct insertion was confirmed by restriction enzymatic digestion using *Eco*RI and *Pvu*II on plasmid DNA isolated from single colonies.

4.1.3 Construction of the dual target plasmid

The plasmids pIRMM-0067 and pIRMM-0089, containing the *hmg* and 98140, respectively, specific sequences, were both digested using *Hin*dIII and *Xba*I restriction endonucleases; the 98140 target fragment was ligated into the linearised *hmg*-containing vector pIRMM-0067. *E. coli* Top 10 cells were then transformed with the ligation products. The transformed cells were plated on LB plates containing 100 µg/mL ampicillin and identified by restriction enzymatic digestion of plasmid DNA extracts from single bacterial colonies. The corresponding plasmid was named pIRMM-0090 (Figure 1) and further tested by real-time PCR. Furthermore, the sequence identity of the cloned fragments was verified by dye terminator cycle sequencing, which confirmed a 100 % sequence identity with the theoretical sequences (Section 8).

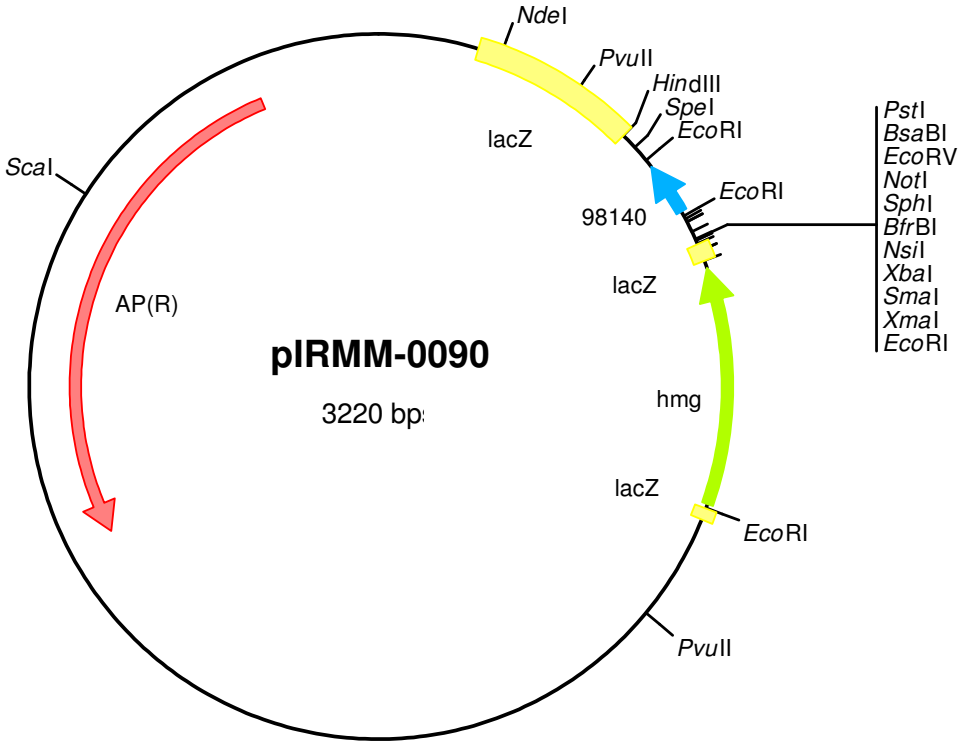

Figure 1: Circular map of pIRMM-0090 representing the 5' insert-to-plant junction and *hmg* inserts as well as the enzymatic restriction sites.

4.2 PURITY OF THE MATERIAL

DNA extracted from positive transformed cells was subjected to endonuclease restriction analysis. The purity of the plasmid pIRMM-0090 was then analysed by agarose gel electrophoresis (Figure 2) and no other DNA bands than those expected after restriction of pIRMM-0090 with *Eco*RI (lane 2), *Hin*dIII/*Xba*I (lane 3), and *Pvu*II (lane 4) could be seen after ethidium bromide staining. This confirmed the correct cloning of the fragments.

Furthermore, following restriction site analysis, no smear and/or RNA band were visible, as demonstrated by agarose gel electrophoresis (Figure 2, lanes 1-3). However, remaining traces of genomic DNA from host bacterial cell or traces of RNA molecules can not be excluded in the final plasmid preparation. Nevertheless, such traces do not affect the real-time PCR measurements and, consequently, the ratio of target sequences, as the used primers and probe are highly specific for the targeted sequences and do not hybridise to other DNA fragments that could be present in the final plasmid preparation. Such traces may introduce, however, a bias in the UV absorbance-based DNA quantification of the plasmid solution, and therefore an erroneous estimation of the number of plasmid copies in the tube. For this reason, the DNA concentration in each tube can only be given as an approximate value.

A BLASTN 2.2.24+ analysis of the *hmg*- and 98140-specific detection PCR primers did not reveal more than 70 % nucleic acid sequences identity with the genomic DNA of *E. coli* from the NCBI database (data not shown). Therefore, specific PCR amplification occurring due to a potential contamination with bacterial genomic DNA is not possible. In addition, real-time PCR analyses of each cloned target (*i.e.* 98140 and *hmg*), performed on either genomic DNA extracted from seed powder or plasmid DNA, had comparable PCR efficiencies, demonstrating no significant effect of potentially contaminating bacterial genomic DNA on the real-time PCR measurements. Furthermore, these observations were supported by

digital PCR [5] experiments that confirmed also the expected ratio between the two target sequences (the measured DNA copy number ratio of the 98140 and *hmg* targets in ERM-AD427 and its expanded uncertainty U (k = 2) is 0.93 ± 0.14). It can be, therefore, concluded that no contamination with external genomic DNA or a significant amount of RNA molecules occurred during the plasmid preparation.

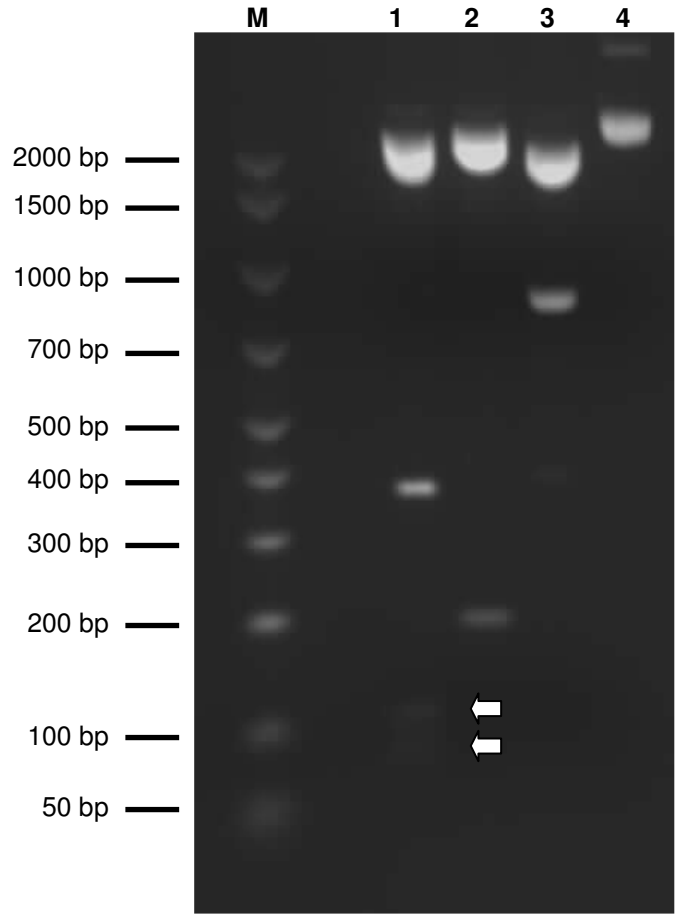

Figure 2: Restriction analysis of pIRMM-0090. Lane M: molecular DNA marker (AmpliSize™ 50-2000 bp ladder, Bio-Rad Laboratories, Hercules, CA, US); lane 1: pIRMM-0090 restricted with *EcoR*I (expected fragments: 2683 bp, 367 bp, 98 bp, 72 bp [hardly visible on the photo, white horizontal arrows indicate the positions of smaller fragments]); lane 2: pIRMM-0090 restricted with *Hind*III/*Xba*I (expected fragments: 3028 bp, 192 bp); lane 3: pIRMM-0090 restricted with *Pvu*II (expected fragments: 2364 bp, 856 bp); lane 4: pIRMM-0090 uncut.

Despite the fact that the enzymatic restriction conditions were chosen to allow for a full digestion of the intermediate plasmids used for the assembly of pIRMM-0090, it is very difficult to prove that all plasmids were indeed fully digested, as traces of undigested plasmids will not be visible after gel electrophoresis and ethidium bromide staining.

The *E. coli* cells could be transformed with three types of plasmids: pIRMM-0090 present in large amount and traces of both undigested pIRMM-0067 and pIRMM-0089. However, as these three synthetic plasmids have the same replication origin (oriV) from ColE1 plasmid, they belong to the same incompatibility group (incQ). As a result, the transformed bacterial clones can only bear one single plasmid. As the plasmid production was started from a separate colony, only one type of plasmid can be present in one colony. We can therefore

conclude that each single bacterium extracted from one colony contains only one type of plasmid[1].

As additional proof of purity, the plasmid DNA isolated from the transformed *E. coli* cells was sequenced completely to verify that both target DNA sequences were present and correctly cloned. The sequence analysis did not reveal the presence of a mixed population of plasmids.

4.3 PROCESSING OF THE MATERIAL

Fifty mL of LB media containing 100 µg/mL of ampicillin were inoculated with a single colony of *E. coli* containing the plasmid pIRMM-0090 and shaken at 200 rpm overnight at 37 °C. The bacterial cells were harvested by centrifugation at 4 °C and resuspended in 50 mmol/L Tris-HCl buffer,10 mmol/L EDTA, pH 8.0. The bacterial pellet was lysed in the presence of RNase, following the recommended protocol QIAprep spin Miniprep kit (QIAGEN Benelux B.V., Venlo, NL). The plasmid DNA was purified on a silica membrane and eluted in 10 mmol/L Tris-HCl buffer, pH 8.5. The resulting DNA solution was stored at -20 °C until further use.

The plasmid DNA concentration and purity of the preparation were measured by UV spectrophotometry and fluorometry. A DNA mass concentration and standard deviation of (191 ± 12) µg/mL were obtained using the PicoGreen® dsDNA quantitation kit (Molecular Probes Inc., Eugene, OR, US). The purity of the nucleic acid preparation was assessed spectrophotometrically by comparing the UV absorbance of the sample at 260 nm to that at 280 nm. Taking into account the generally accepted mean value of the extinction coefficient for dsDNA at 260 nm and 280 nm [7], pure nucleic acid samples are expected to have an A_{260}/A_{280} ratio approaching 2.0 and an A_{260}/A_{230} ratio above 2.0. The A_{260}/A_{280} and A_{260}/A_{230} ratios measured for the plasmid solution were 1.89 and 2.25 respectively, indicating a sufficient DNA purity; however, such values do not exclude traces of contaminating proteins [8].

The measured DNA mass concentration was used to calculate the number of copies of plasmid per volume. For this estimation, the following formulas were applied:

1 g of DNA contains x copies of a plasmid
with $x = N_A/[2 \cdot M \cdot S]$ (1)

where, N_A = Avogadro constant (mol^{-1}),
M = mean molar mass of a nucleotide (g/mol),
S = size of the plasmid molecule (number of nucleotides per plasmid DNA copy),
x = number content (number of copies of plasmid/g).

[1] The synthetic vectors (pUC18 and pCR®2.1) used in our cloning strategy were chosen as being high copy vectors from the same incompatibility group (incQ). These plasmids have their own oriV and are able to replicate independently of the host chromosome. A bacterial host cannot, however, contain different plasmids that have the same mechanisms of replication, because the control of the replication (in Gram-negative bacteria) is exercised through trans-acting molecules (theta replication mode). The inevitable consequence of this is that one of the plasmids would eventually be lost from the cell simply as a result of random partitioning of plasmids into daughter cells during cell division. Thus, the plasmids would appear to be incompatible. Two or more plasmids from the same incQ cannot coexist in the same cell [6].

The number of plasmids per volume can be calculated by measuring the DNA concentration of the solution and using the following formula:

$$\text{copy number concentration [cp/µL]} = \frac{\text{DNA mass concentration [g/µL]} \cdot N_A\text{[bp/mol]}}{2 \cdot M\text{[g/mol]} \cdot S\text{[bp/cp]}} \quad (2)$$

A solution containing approximately 2×10^6 cp/µL of the plasmid pIRMM-0090 corresponding to a concentration of 6.6 pg/µL was prepared by diluting the stock plasmid solution in a background of ColE1 plasmid DNA (product number D9683, Sigma-Aldrich, Bornem, BE) that amounted to a final concentration of 1 ng/µL in 1.0 mmol/L Tris-HCl, 0.01 mM EDTA, pH 8.0 (TE buffer).

4.4 FILLING OF TUBES

The above plasmid solution was sterilised by filtration through a 0.2 µm pore-sized filter and filled manually in pre-labelled, high recovery, medical-grade polypropylene tubes with 500 µL plasmid solution, under sterile conditions. Each tube contains approximately 10^9 copies of the ERM-AD427 plasmid, which corresponds to about 3.3 ng of DNA.

The tubes were placed in cardboard boxes each containing 100 tubes. Tubes for the short- and long-term stability studies, and additional characterisation were selected randomly from the entire batch. Each box was then sealed under light vacuum in a plastic pouch and frozen either at (-20 ± 5) °C or at (-70 ± 10) °C.

Each tube was identified by a numbered label as shown in Figure 3.

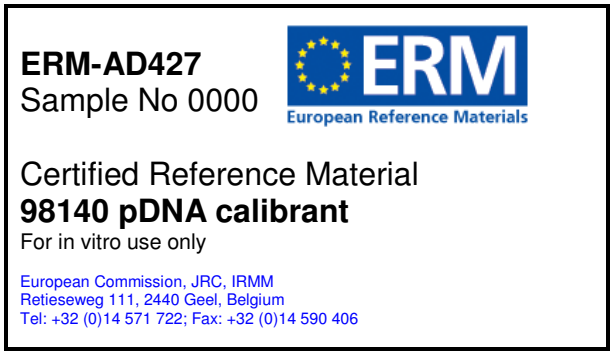

Figure 3: Prototype label for ERM-AD427.

4.5 DISPATCHING OF TUBES

Tubes containing the processed ERM-AD427 were dispatched to the participants to the interlaboratory comparison launched to evaluate the suitability of calibrants (Section 8.3.1). Unprocessed samples containing purified plasmid pIRMM-0090 (*i.e.* without background ColE1 plasmid DNA) in nuclease-free water were dispatched to one independent sequencing service company to determine the complete DNA sequence of the plasmid. All samples were packed in containers filled with dry ice.

5 PROCEDURES

5.1 METHOD USED FOR THE STABILITY STUDY

Simplex real-time PCR targeting an 80 bp fragment of the 98140 event and a 79 bp fragment of the *hmg* gene was used to identify and quantify the amount of both target sequences present in the plasmid DNA. The setup of the real-time PCR was done according to the procedure described in the method validated by EURL-GMFF for the event specific quantification of the 98140 maize [9].The real-time PCR was performed using an ABI 7900HT instrument and primer pairs and labelled TaqMan® probes specific for the 98140 event or for the maize reference gene *hmg* [9], following the TaqMan® Universal PCR Master Mix protocol (Applied Biosystems, Foster City, CA, US) [10].

5.2 METHODS USED FOR CHARACTERISATION

5.2.1 Gel electrophoresis

The restriction pattern of plasmid DNA samples was analysed by gel electrophoresis, using a Sub-Cell GT agarose gel electrophoresis system (Bio-Rad Laboratories, Hercules, CA, US) and 2 % mass fraction agarose gel containing 0.5 µg/mL ethidium bromide. Ten µL aliquots of each enzymatic restriction mixture were electrophoresed at constant voltage (100 V) for 45 min in TBE buffer (90 mmol/L Tris-HCl, 90 mmol/L boric acid, 2 mmol/L EDTA, pH 8.0). DNA was visualised by UV light and photographed using a GeneGnome system (Syngene, Leusden, NL).

5.2.2 Fluorometry and UV spectrometry

The extracted plasmid DNA was quantified using the PicoGreen® dsDNA Quantitation Kit (Molecular Probes, Eugene, OR, US) according to the manufacturer's instructions, using a lambda DNA standard solution. The purity of the DNA in the solution was analysed by measuring the UV absorbance at 230, 260 and 280 nm on a UV-Vis spectrophotometer (NanoDrop ND-1000, NanoDrop Technolgies Inc., Wilmington, DE, US). The ratios of the absorbances at 260 and 280 nm (A_{260}/A_{280}) and of the absorbances at 260 and 230 nm (A_{260}/A_{230}) were calculated to provide an estimation of the purity of the extracted DNA.

5.2.3 DNA sequencing

The plasmid pIRMM-0090 was sequenced by two independent laboratories: Joint Research Centre, IRMM (Geel, BE) and Eurofins Medigenomix GmbH (Martinsried, DE).

The sequencing was performed at IRMM on an ABI Prism® 3130*xl* Genetic Analyser (Applied Biosystems, Lennik, BE) using the BigDye® Terminator v1.1 cycle sequencing kit protocol. Cycle sequencing utilises successive rounds of denaturation, annealing and extension in a thermocycler to create a linear amplification of extension products. With dye terminator labelling, each of the four dideoxy terminators is tagged with a different fluorescent dye. The 3130*xl* RapidSeq36_POP7 run module was used, and the samples were analysed using the ABI Sequencing Analysis Software v5.2 Patch 2.

Eurofins Medigenomix GmbH (Martinsried, DE) performed double strand sequencing by primer walking of the entire pIRMM-0090 plasmid, on an ABI Prism® 3130*xl* instrument. All sequences were generated using BigDye-terminator chemistry (version 3.1) of Applied Biosystems (Foster City, CA, US) following standard protocols. For PCR reactions Primus 96 HPL Thermalcyclers (MWG AG, Ebersberg, DE) were used. Sequencing reaction cleanup was done by gel filtration through Performa DTR 96 well plates (Edge Biosystems, Gaithersburg, MD, US). Finally all reactions were run on ABI3730*xl* capillary sequencers.

The sequence quality values which are a transformed estimate of the probability of correctness (1 – probability of error) are represented in a log scale from 0 to 50 for single strand sequencing. The use of a log-transformed error probability facilitates working with error rates in the interval of most importance (very close to 0). The quality value *q*, which is the per-base estimate of the accuracy of base calling (*i.e.* assignment of A, C, G, T, or N to every base), is defined by the following equation:

$$q = -10 \cdot \log_{10} z \qquad (3)$$

where *z* is the estimated error probability for a base.

The sequences generated by Eurofins Medigenomics (Martinsried, DE) had a quality value of at least 84 which means that the estimated probability for each base to be incorrect was less than 1/250 000 000.

5.3 METHODS USED FOR THE SUITABILITY STUDY

A study designed to perform the sequence identification and quantification of the *hmg* and 98140 targets present in ERM-AD427, and for the evaluation of the analytical behaviour of the DNA calibrants used in the quantification of the 98140 event in maize seed powder was included as part of the interlaboratory comparison organised to assess the DNA copy number ratio of ERM®-BF427c [11]. The sequence identification and quantification of the 98140 transgenic target was performed using an event-specific method targeting a 80 bp fragment of the 5'-plant junction region and a 79 bp fragment of the *hmg* gene used as normaliser. The final forward and reverse primer concentrations in the PCR reaction was 400 nmol/L and 500 nmol/L for the *hmg* and 98140 targets, respectively, while the concentration of oligonucleotide probe was 150 nmol/L and 200 nmol/L in the *hmg* and 98140 amplification reactions, respectively. TaqMan® Universal PCR analyses were carried out according to the manufacturer's instructions (Applied Biosystems, Lennik, BE) with a thermal profile consisting of an initial denaturation step at 95 °C for 10 min, followed by 45 amplification cycles of 15 s at 95 °C and 1 min at 60 °C. The TaqMan® Universal PCR MasterMix contained AmpliTaq Gold® DNA polymerase and AmpErase® UNG. The first one is known to allow for a better yield, whereas the second ensures a more robust 5' nuclease assay than AmpliTaq® DNA polymerase and protects against subsequent reamplifications from PCR products, minimising as such the carry-over contamination. The MasterMix also contained a passive reference dye to correct for inter-well signal variation caused by slight differences in the reaction volume.

Five µL of template DNA solution was added to 20 µL master mix containing the appropriate primers and probe for the simplex real-time PCR. Both 98140 and *hmg* probe were 5′-labelled with 6-carboxylfluorescein (6-FAM). The fluorescence is recorded on-line (in real-time) during the PCR amplification process and its intensity is proportional to the number of target DNA sequences.

For the purpose of comparing the behaviour of pDNA and gDNA, three types of DNA were used: plasmid DNA ERM-AD427, genomic DNA extracted from seed powder (gDNA$_s$) certified for its mass fraction content of 98140 (ERM-BF427c) and genomic DNA extracted from 98140 maize plant leaves (gDNA$_\ell$). Genomic DNA from 98140 plant leaves was obtained at IRMM by DNA extraction of maize leaves harvested from germinated 98140 maize seeds, heterozygous for the GM event, using the protocol for automated DNA extraction and the Chemagic DNA Plant Kit (Chemagen Biopolymer-Technologie AG, Baesweiler, DE) following the manufacturer's protocol. For the extraction of genomic DNA from seed powder, samples of the CRM ERM-BF427c were used. The plasmid DNA, genomic DNA from plant leaves extracted at IRMM and the maize powder CRM ERM-BF427c were shipped to the participating laboratories, on dry ice. For each data set, the

genomic DNA from seed powder was obtained by each participant using one of the three DNA extraction methods: i) a modified cetyltrimethylammonium bromide (CTAB) method [12], ii) the DNeasy plant mini kit (Qiagen, Benelux B. V., Venlo, NL), and iii) GENE*Spin* DNA kit (GeneScan Analytics GmbH, Freiburg, DE). The extraction methods used in the study are described in detail in the certification report on the DNA copy number ratio of ERM®-BF427c [11].

6 HOMOGENEITY STUDY

6.1 HOMOGENEITY ASSESSMENT

The certified value for ERM-AD427 is expressed as number of DNA fragments per pIRMM-0090 plasmid. As this quantity defines the structure (DNA sequence) of a pure substance (pIRMM-0090), the homogeneity of the material is not considered in the estimation of the uncertainty of the certified value.

6.2 MINIMUM SAMPLE INTAKE

In the frame of the short-term stability study and interlaboratory comparison addressing the analytical behaviour of pDNA and gDNA with respect to the real-time PCR quantification of the 98140 event in maize (Sections 7.1 and 8.3), it was shown that using 50 µL ERM-AD427 solution for the preparation of a calibration curve by serial dilution led to reliable PCR results. The validity of the PCR measurements was, however, not tested with smaller starting volume intakes. Therefore, the recommended minimum sample intake for setting up a calibration curve for real-time PCR, using the described method [9], is 50 µL.

7 STABILITY

7.1 SHORT-TERM STABILITY STUDY

7.1.1 Design of the short-term stability study

The short term stability of ERM-AD427 was evaluated by analysing 5 tubes stored at (-20 ± 5) °C, (4 ± 3) °C and (60 ± 5) °C for 2 and 4 weeks. Three replicates from each tube were analysed (N = 5, n = 3) at several dilution levels. The same number of tubes were stored at (-70 ± 10) °C as reference. Each tube was analysed in triplicate by simplex real-time PCR to reveal changes in the amount of the two sequences present in the plasmid due to the different test temperatures and storage times.

7.1.2 Results of the short-term stability study

The ratio of both sequences was analysed and did not indicate any significant change at (-20 ± 5) °C, (4 ± 3) °C or (60 ± 5) °C (Figure 4). DNA copy number ratios measured by real-time PCR followed a normal distribution at each of the three chosen storage temperatures. Scrutinising the data obtained, the single Grubbs test revealed that the data set at 4 °C contains one outlier at 95 % confidence level. As no technical reasons for the outliers could be identified, the outlying data was retained.

Regression analysis was performed for each of the storage temperatures in order to detect any trend in the ratio between targets in relation to the time of storage. A *t*-test showed no significant change over the time period of 4 weeks (95 % confidence level) for the material kept at (-20 ± 5) °C, (4 ± 3) °C and (60 ± 5) °C when testing the plasmids at final copy number concentrations estimated to be between 400 000 cp/µL and 4 cp/µL. However, a certain degree of instability was observed at (60 ± 5) °C after 4 weeks, affecting the ratio between targets in highly diluted samples. After storage at (60 ± 5) °C, 4 cp/µL could not be amplified after 4 weeks, whereas 40 cp/µL was still measurable, but with large variation (data not shown). These effects are probably due to a partial degradation of plasmids exposed to high temperatures.

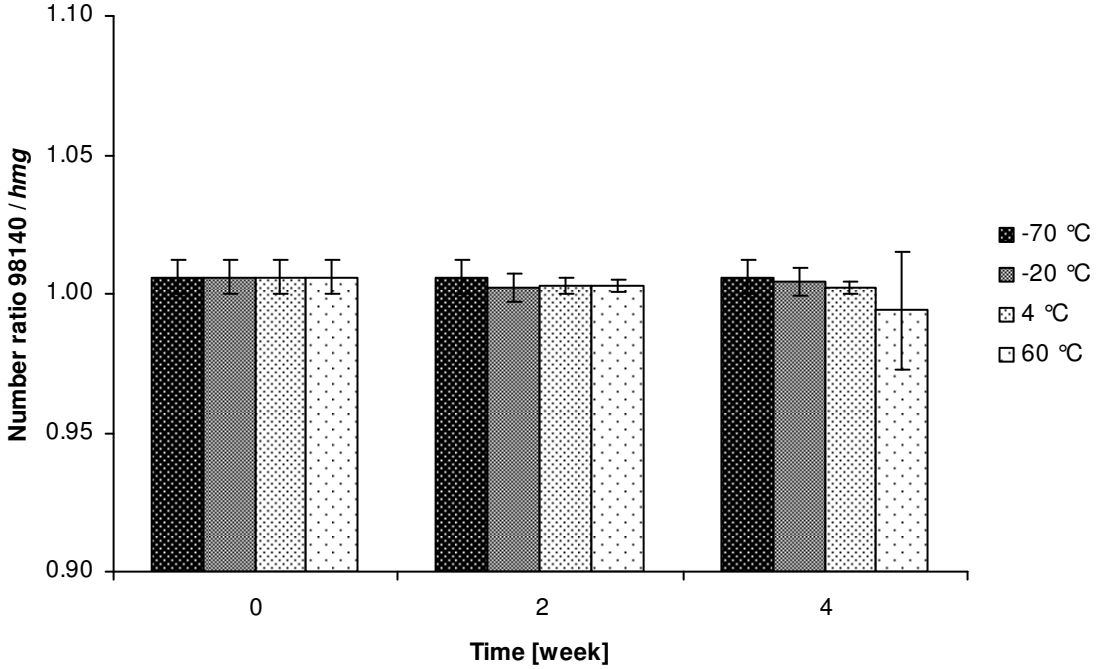

Figure 4: Short-term stability of ERM-AD427 stored at different temperatures for 0, 2 and 4 weeks and analysed by *hmg* and 98140 specific real-time PCR. The columns and bars indicate the mean copy number $\bar{x}$ ± standard deviation s, for N = 5 and n = 3.

7.1.3 Conclusion from the short-term stability study

The short-term stability study performed for ERM-AD427 proved that ERM-AD427 withstands temperatures of up to at least 4 °C for the tested periods of time.

More information about the stability of similar materials has been gained on plasmid DNA materials that were processed using the same isolation and purification protocols, mixed in the same proportion with the same batch of ColE1 background DNA, based on the same vector, stabilised in the same buffer composition and stored in the same type of tubes. Because of the similar processing and identical nature of the DNA, it is reasonable to consider that all materials will have the same stability features.

Data from the isochronous long-term stability study of ERM-AD413 stored at (18 ± 5) °C during 24 months (see details in Section 7.2) indicate also that such materials can be shipped using cooling elements.

7.2 LONG-TERM STABILITY STUDY

7.2.1 Design of the long-term stability study

As mentioned above, ERM-AD427 was produced following the same procedure as for ERM-AD413 [13]. Therefore, to estimate the stability of ERM-AD427 during long-term storage, data obtained in the frame of the ERM-AD413 post-certification monitoring for a period of 24 months was used. Tubes containing ERM-AD413 were stored at (-20 ± 5) °C and (18 ± 5) °C to be analysed at the respective times (Figure 5).

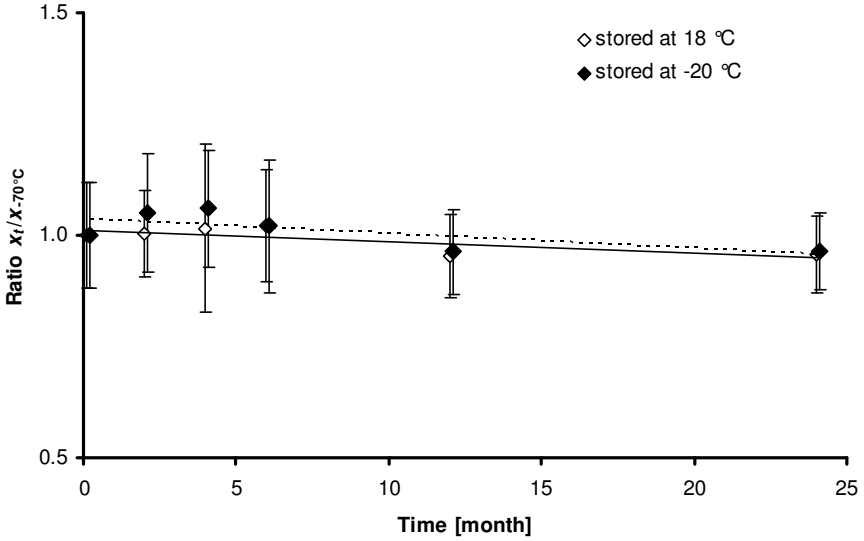

Figure 5: Long-term stability of the plasmid DNA ERM-AD413 (MON 810 calibrant) stored at (-20 ± 5) °C and (18 ± 5) °C for 24 months, based on real-time PCR measurements. The stability is expressed as the ratio between the copy number ratio of MON 810 and *hmg* fragments per plasmid in tubes stored at different temperatures, and the corresponding copy number ratio of tubes stored for the same period at the reference temperature (-70 ± 10) °C. Each bullet corresponds to the mean ± standard deviation *s* of 5 to 17 measurements. The dashed line is the regression line generated on the basis of the (-20 ± 5) °C data points, whereas the full line is the regression line generated on the basis of the (18 ± 5) °C data points.

7.2.2 Conclusions of the long-term stability study

Based on the stability study of ERM-AD413, a minimum shelf-life of 24 months at (-20 ± 5) °C can be ensured for real-time PCR applications. The ratio between the two inserted targets measured by real-time PCR remains unaffected, and the material can be, therefore,

considered as suitable for use in real-time PCR measurements. The total amount of the material present in the tubes does not decrease, as the Ct values for the respective targets remain unchanged upon storage.

The long-term stability study does not show any difference for the material storage at (-20 ± 5) °C or at (18 ± 5) °C, in terms of copy number ratio. We recommend, nevertheless, storing the material at (-20 ± 5) °C, if the material needs to be stored for several months. Furthermore, yearly post-certification monitoring is planned to provide an additional control of the stability of ERM-AD427 and to allow for extending the shelf-life of this CRM.

8 BATCH CHARACTERISATION

8.1 PLANNING

To verify the correct number ratio between the two cloned DNA fragments in the ERM-AD427 calibrant, the full sequence of the dual-target plasmid pIRMM-0090 was determined by two independent laboratories. The DNA sequencing was performed in-house on an ABI Prism® 3130*xl* Genetic Analyser (Applied Biosystems, Lennik, BE) using the BigDye® Terminator v1.1 Cycle Sequencing kit. Additionally, Eurofins (Medigenomix GmbH, Martinsried, DE) company was asked to provide the full sequence of ERM-AD427. The plasmid was sequenced on both strands in order to ensure an accurate determination by a two fold-coverage of the generated sequences, as well as a complete characterisation of the molecular composition of plasmid DNA.

The resulting sequences were compared with each other and submitted to the GenBank® database (NIH, Bethesda, MD, US) for BLASTN homology searches [14]. The determined DNA sequences were identical and confirmed the correct cloning of the two fragments. Furthermore, the correctness of the two sequences was proven by the 100 % identity with the published sequences. The ERM-AD427 plasmid could be accurately sized and consists of 3220 bp (Figure 1). The DNA sequencing also confirmed that each plasmid contained single *hmg* and 98140 fragments. The uncertainty related to the sequence determination can be considered negligible, as the probability to report a wrong base (error probability) was calculated to be less than 1/250 000 000, according to the laboratory performing the complete sequencing. As a clear consequence, the structurally defined ratio between the numbers of 5'-insertion-specific DNA and *hmg* fragments in the plasmid pIRMM-0090 is 1.

8.2 ASSIGNMENT OF THE CERTIFIED VALUE

The material is certified to contain one 80 bp 5' insert-to-plant junction fragment and one 79 bp *hmg* fragment per pIRMM-0090 plasmid[2] based on DNA sequencing. This certified value is based on the DNA sequence of pIRMM-0090 provided by two independent laboratories.

8.3 SUITABILITY STUDY

Twenty-one laboratories were selected on the basis of proven experience and quality management system in place to investigate the analytical behaviour of the calibrants used for calibration of real-time PCR measurements. Within the same interlaboratory comparison, the determination of the copy number ratio related to the ERM-BF427c matrix was performed using simplex real-time PCR; a total of 36 analyses were requested that resulted in 140 real-time PCR experiments (a detailed description of the methodology of the study is given in [11]). Within one data set of the study, each laboratory carried out two independent analyses, on two different days. Consequently, the experimental setup on each day was based on two calibration curves, namely, transgenic and endogeneous calibration curves, each of them including five concentration levels. The PCR efficiencies as well as the linearity of the calibration curves were calculated on the basis of serial dilutions in 1 mmol/L Tris, 0.01 mmol/L EDTA, pH 8.0 buffer of the plasmid calibrant (ERM-AD427), dilutions of gDNA extracted from leaves from germinated 98140 maize seeds, labelled in the Figures and Tables as gDNA$_{\ell}$, as well as dilutions of gDNA extracted from ERM-BF427c (containing 20 g of 98140 maize seeds per kg of maize) and labelled as gDNA$_{s}$. To evaluate the analytical behaviour of the plasmid and genomic DNA, these two parameters were compared and statistically analysed.

[2] The certified value expressed as number of DNA fragments per plasmid DNA molecule is an entitic number.

The first parameter was the PCR efficiency (ε) estimated for both transgenic and endogenous targets, using the three DNA types. The PCR efficiencies were only compared if the respective data sets passed the selection criteria defined beforehand, namely the coefficient of determination (R^2) of the calibration curve, and PCR efficiency (estimated on the basis of the slope of the calibration curve). These selection criteria were applied in order to avoid interferences of technically weak results generated by the participating laboratories [15, 16]. Firstly, a R^2 below 0.98 was not accepted within the study as it may reflect erroneous dilutions or inappropriate PCR amplification. Secondly, the PCR efficiencies interval was defined on the basis of pDNA and gDNA efficiencies (both materials used as calibrants in the study). The means of the PCR efficiencies were calculated for the endogeneous and transgenic targets using either the pDNA or gDNA calibrant. Four intervals were accordingly generated based on mean ± 1 *s*. The minimum and maximum values of the resulting cut-off points were then used to define the lower and higher limits of the PCR efficiency of the study, *i.e.* 87 and 106 % respectively. These values were consistent with the performance of the simplex real-time PCR method for quantification of the 98140 maize event observed during in-house performed studies and from results obtained by the participants in the interlaboratory comparison.

The PCR efficiencies estimated for both endogeneous and transgenic targets were comparable when using pDNA and gDNAℓ calibrants (96.9 and 95.2 % versus 96.5 and 95.6 % for the pDNA and gDNA calibration curves of the taxon- and event-specific detection methods, respectively) (Table 1). No significant differences between PCR efficiencies of pDNA and gDNAℓ calibrants were found for the endogeneous and transgenic targets, *hmg* (p = 0.49, α = 0.05) (Figure 6A) and 98140 (p = 0.58, α = 0.05) (Figure 6B) on simplex real-time PCR detection methods.

The distributions of the various PCR efficiencies for pDNA/gDNAℓ calibrant and gDNAs were also compared and showed a large overlap for both targets for the three DNA types (Figure 6). The PCR efficiencies of the gDNAs calibration curves were always larger than the PCR efficiencies obtained with pDNA and gDNAℓ. Except for the difference between the PCR efficiencies of the transgenic target for gDNAℓ and gDNAs, noted as statistically not significant (p = 0.054, α = 0.05), all other comparisons led to significantly different results for the 98140 target sequence (Figure 6 and Table 1).

Table 1: Comparison of the real-time PCR efficiencies of gDNA extracted from leaves and seeds, and pDNA; N_{ds} is the number of accepted data sub-sets under repeatability conditions, *s* is the standard deviation.

Target sequence	Mean PCR efficiency ± *s* [%]		
	ERM-AD427 pDNA	Seeds gDNAs	Leaves gDNAℓ
hmg	96.9 ± 2.9 (N_{ds} = 61)	98.5 ± 4.0 (N_{ds} = 84)	96.5 ± 3.2 (N_{ds} = 66)
98140	95.2 ± 4.2 (N_{ds} = 58)	97.3 ± 5.6 (N_{ds} = 72)	95.6 ± 3.9 (N_{ds} = 56)

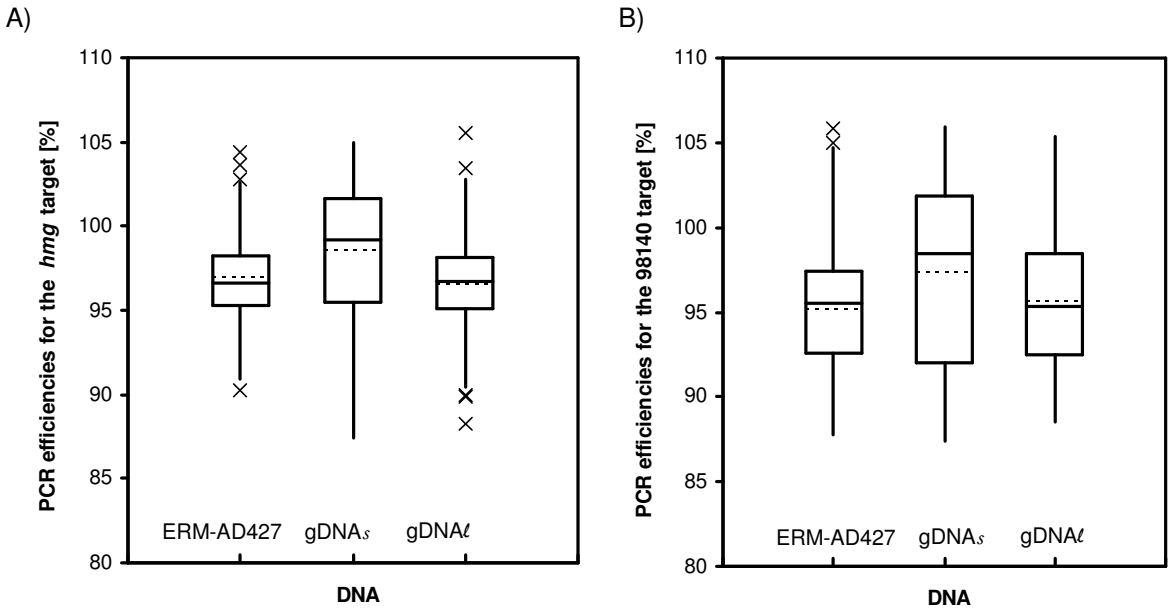

Figure 6: Box-and-whisker diagram illustrating the PCR efficiencies for the *hmg* (A) and 98140 (B) target genes, based on the calibration curves performed using either ERM-AD427 calibrant or gDNA isolated from 98140 seeds or 98140 leaves. The bottom and top of the box are the 1st and 3rd quartiles, respectively; the line near the middle of the box is the median. The ends of the whiskers are determined by subtracting 1.5 times the interquartile range (IQR) from the 1st quartile, and adding 1.5 times the IQR to the 3rd quartile, respectively. The dashed line corresponds to the mean value of each group, whereas outlier values (95 % confidence level) are marked with x.

The second parameter studied was the coefficient of determination (R^2) that provides information about the fitting of data to a linear calibration curve obtained for both targets, using the three types of DNA (Figure 7 and Table 2).

Table 2: Comparison of the coefficients of determination (R^2) of gDNA extracted from leaves or seeds and pDNA; N_{ds} is the number of accepted data sub-sets under repeatability conditions, *s* is the standard deviation.

Target sequence	Mean $R^2 \pm s$		
	ERM-AD427 pDNA	Seeds gDNA*s*	Leaves gDNA*ℓ*
hmg	0.999 ± 0.001 (N_{ds} = 61)	0.997 ± 0.004 (N_{ds} = 84)	0.999 ± 0.001 (N_{ds} = 66)
98140	0.994 ± 0.004 (N_{ds} = 58)	0.995 ± 0.004 (N_{ds} = 72)	0.999 ± 0.002 (N_{ds} = 56)

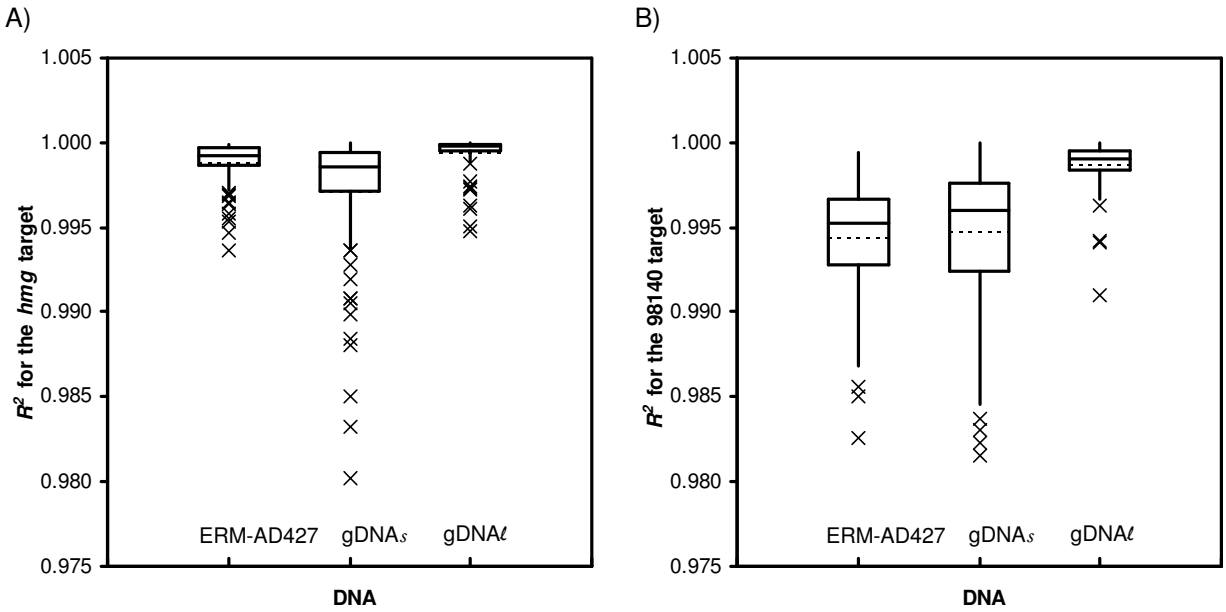

Figure 7: Box-and-whisker diagram representing the coefficients of determination (R^2) for *hmg* (A) and 98140 (B) target genes, based on the calibration curves performed using either ERM-AD427 calibrant or gDNA extracted from 98140 seeds or 98140 leaves. A description of a box-and-whisker diagram is given in Figure 6.

A comparison of the coefficients of determination of pDNA and gDNA*ℓ* calibration curves showed no major influence of the type of the DNA calibrant in the case of the endogenous target (Figure 7A). For the transgenic target, the distribution of the coefficients of determination displayed a large variation (Figure 7B). However, the coefficients of determination corresponding to the gDNA*s* dilution curves distributed closer to those of the pDNA calibrant in the case of both targets.

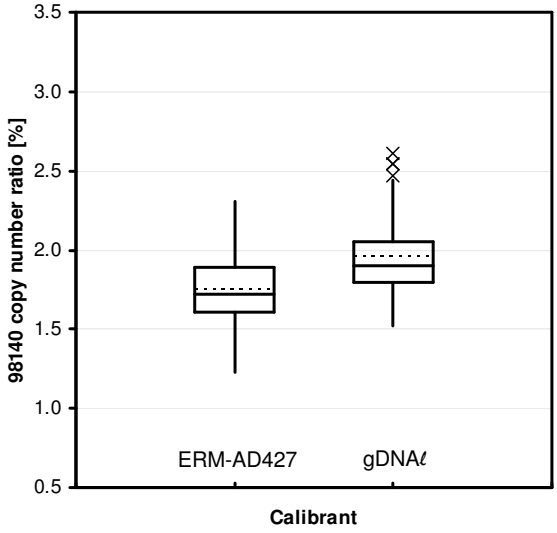

Figure 8: Box-and-whisker diagram representing the 98140 copy number ratio in ERM-BF427c using either ERM-AD427 or gDNA extracted from 98140 leaves as calibrant. A description of a box-and-whisker diagram is given in Figure 6.

Finally, the 98140 GM copy number ratios of ERM-BF427c (20.0 g/kg), measured using either ERM-AD427 or gDNA*ℓ* as calibrants, were compared (Figure 8 and Table 3). To do that, the DNA copy number ratios obtained per day from accepted data sets were evaluated. The data sets from both calibrants follow a normal distribution. Although the GM copy number ratios obtained for ERM-BF427c by the two different calibrants overlap, the mean GM copy number ratios are very different. Single factor ANOVA confirms that the data set calibrated with ERM-AD427 is significantly different from the data set calibrated with

gDNAℓ from leaves (p = 7.6 × 10^{-4}, α = 0.05). Therefore, the GM copy number data calibrated with ERM-AD427 or gDNAℓ from leaves cannot be pooled.

Table 3: GM content in ERM-BF427c expressed in copy number ratio and calibrated with either ERM-AD427 or gDNAℓ extracted from 98140 leaves; N_{ds} is the number of accepted data sub-sets, s is the standard deviation.

	DNA copy number ratio ± s [%]	
	ERM-AD427 pDNA	Leaves gDNAℓ
ERM-BF427c	1.75 ± 0.27 (N_{ds} = 40)	1.96 ± 0.27 (N_{ds} = 44)

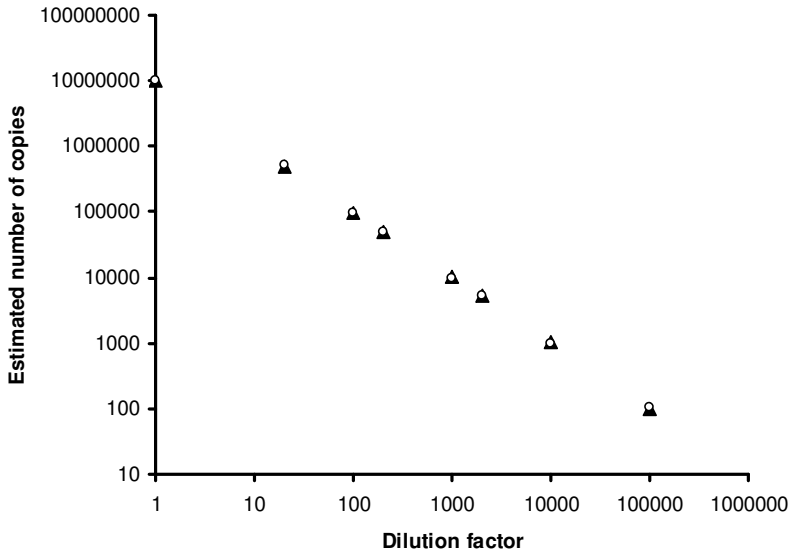

Figure 9: Comparison of the estimated number of copies of the *hmg* (○) and 98140 (▲) target sequences by the simplex real-time PCR method applied for a serial dilution of ERM-AD427 (N = 20) in TE buffer. The copy numbers were estimated by DNA quantification measurements of the initial DNA mass concentration corresponding to the unprocessed plasmid pIRMM-0090, using the PicoGreen® dsDNA quantitation kit.

In the study reported here, statistical analyses showed that pDNA and gDNAℓ calibrants behave in a similar way with respect to the PCR efficiencies of the transgenic and endogeneous target sequence (Figure 6). The individual PCR efficiency of each target sequence has, however, a significant impact on GM quantification by real-time PCR. Therefore, the effect of a small difference in PCR efficiencies of the transgenic and endogeneous targets on GM quantification by real-time PCR can generate a rather large difference in copy number due to the exponential nature of the PCR amplification. Consequently, such a small difference in PCR efficiencies of gDNAℓ and pDNA calibrants may explain the difference between the GM copy number ratios observed when the mean values are compared (Table 3).

Based on this study, it can be concluded that both calibrants, ERM-AD427 pDNA and gDNAℓ extracted from plant leaves, are equally suited to calibrate the real-time PCR method applied here. However, the user should be aware that the choice of the calibrant influences the measured DNA copy number ratio and leads to significantly different results (Table 3).

Due to the biology of maize, it is impossible to establish a reliable DNA copy number ratio based on the GM mass fractions. Using the pDNA calibrant ERM-AD427 or the gDNAℓ

calibrant from leaves might not lead to a true value of the measured DNA copy number ratio.

The results of the suitability study indicate that the most suitable approach is to set a reference system based on the pDNA calibrant. This approach is herein described for the determination of the DNA copy number ratio, together with the GM quantification method validated by EURL-GMFF [9], and leads to the comparability of the measurement results in GM quantification.

As shown in Figure 9, ERM-AD427 can be diluted down to 1/100000 in TE buffer, and can be easily quantified for both targets.

9 CERTIFIED VALUES AND UNCERTAINTY

9.1 CERTIFIED VALUES

The plasmid calibrant is characterised by the number of each specific fragment per pIRMM-0090 plasmid. The cloned DNA fragments, for which ERM-AD427 is certified, *i.e.* 98140 event- and *hmg*-specific sequences, were found to be identical to the respective PCR targets published with the validated methods for the quantification of the 98140 maize, by EURL-GMFF [9]; each fragment is certified to be present as one single copy per pIRMM-0090 plasmid. Based on the sequence and purity assessment, the number ratio between the two targets is equal to 1.

9.2 UNCERTAINTY EVALUATION

Based on the sequence analyses, it can be concluded that the 98140 event and *hmg* DNA fragments are present as a single copy per pIRMM-0090 plasmid. Two independent laboratories provided exactly the same sequences, which also showed a 100 % sequence identity with the expected sequences as published in the NIH genetic sequence database (GenBank®). The DNA sequencing performed by forward and backward sequencing (on both strands) generated the correct sequence for the whole plasmid, *i.e.* 3220 bp analysed. The uncertainty on the sequencing under these conditions can be considered as negligible. The estimated error probability of the sequence identification of each fragment is smaller than 0.00003 %.

10 METROLOGICAL TRACEABILITY

The certified value is expressed as a number of DNA fragments per plasmid. ERM-AD427 is certified for the number of 80 bp fragments of 5' insert-plant specific (98140 event) DNA and the number of 79 bp *hmg* DNA fragments in the plasmid pIRMM-0090. This number is determined on the basis of the full sequence of the plasmid pIRMM-0090, and is traceable to the SI.

The purity of the preparation and the number of each cloned fragment was confirmed by endonuclease restriction. The use of identical vectors to construct pIRMM-0090 originating from single colonies ensures the presence of only one type of plasmid bearing one copy of the 98140 event and the *hmg* DNA fragments each. The sequence analysis confirmed the presence of only one type of plasmid in the pIRMM-0090 preparation.

End-point PCR followed by agarose gel electrophoresis (data not shown) as well as digital PCR were used to further investigate the purity of ERM-AD427. No evidence for contamination with respect to the certified properties was found. However, the ability of the methods applied in this study to investigate the purity of the material was limited and can only ensure a purity of at least 90 %.

11 COMMUTABILITY

The two calibrants tested during the suitability study, pDNA ERM-AD427 and gDNA$_\ell$ extracted from plant leaves, led to significant differences in the measured copy number ratio values of ERM-BF427c (Table 3). Commutability [17] problems should be considered for the copy number ratio measurements of DNA extracted from food and feed samples. It could not be proven during the suitability study that one of the calibrants (pDNA/gDNA$_\ell$) behaves more similar to the gDNA$_s$ extracted from seed powder.

However, based on the traceability chain described above, as well as on additional practical reasons, which include full sequence characterisation, reproduction of additional batches of calibrant and availability, the pDNA ERM-AD427 is selected as the calibrant of choice to be used for the calibration of the event-specific method for the quantification of the 98140 event. The user should, therefore, be aware that values measured with the 98140 event-specific real-time PCR method and calibrant ERM-AD427 are reproducible. However, values obtained with this measuring system may not be close to the true value.

12 INSTRUCTIONS FOR USE

12.1 INTENDED USE

The ERM-AD427 is intended to be used as calibrant exclusively with the method for quantification of the maize 98140 event, validated by EURL-GMFF [9]. ERM-AD427 can therefore also be used for the quantification of the 98140 event present in food and feed products containing such event.

When using ERM-AD427 for the calibration of real-time PCR for the quantification of the maize 98140 event, the value estimated from the real-time PCR measurement and its related uncertainty should be taken into consideration.

The recommended minimum sample intake is 50 µL to perform a dilution series (see Annex 2). A minimum sample intake of 5 µL is recommended to be used per real-time PCR assay.

12.2 HANDLING

The plasmid tube should be opened and handled under a laminar flow to reduce the risk of contamination. ERM-AD427 has a copy number concentration of approximately 2×10^6 cp/µL of the pIRMM-0090 plasmid calibrant. Dilution series should always be prepared freshly prior to a real-time PCR run. TE buffer (1 mmol/L Tris buffer, 0.01 mmol/L EDTA, pH 8.0) should be used as plasmid dilution buffer. A proposed example for the preparation of calibration curves is given in Annex 2 (see also ERM Application Note 5 [18]).

ERM-AD427 can be treated with *Hind*III or *EcoR*V restriction endonucleases (unique restriction sites in the plasmid), which keep the two cloned targets present in pIRMM-0090 intact. Restriction with *Hind*III will keep the 98140 and *hmg* fragments next to each other, whereas *EcoR*V will leave the cloned targets at both extremities of the linearised pIRMM-0090.

12.3 TRANSPORT AND STORAGE

The ERM-AD427 shall be dispatched with cooling elements and has to be kept at 4 °C or lower temperature upon arrival. However, for long-term storage, keeping the material at -20 °C is recommended. The serial dilutions of the plasmid should be prepared freshly prior to the real-time PCR measurement.

ACKNOWLEDGEMENTS

The authors would like to thank E. de Andrade Silva for technical assistance, L. Deprez, W. Meyer, B. Jeynov and T. Linsinger from IRMM for numerous and fruitful discussions, and E. de Vos and W. Meyer from IRMM for the reviewing of the certification report, as well as the experts of the Certification Advisory Panel 'Biological Macromolecules and Biological/Biochemical Parameters', R. Dybkaer (Frederiksberg Hospital, Frederiksberg, DK), A. Heissenberger (Umweltbundesamt/Environment Agency Austria, Vienna, AT) and U. Örnemark (Emendo Dokumentgranskning Handelsbolag, Hillared, SE) for their critical comments.

REFERENCES

1. Regulation (EC) No 1830/2003 of the European Parliament and of the Council of 22 September 2003 concerning the traceability and labelling of genetically modified organisms and the traceability of food and feed products produced from genetically modified organisms and amending Directive 2001/18/EC. Official Journal of the European Union 2003, L(268):24-28.
2. Commission Recommendation of 4 October 2004 on technical guidance for sampling and detection of genetically modified organisms and material produced from genetically modified organisms as or in products in the context of Regulation (EC) No 1830/2003. Official Journal of the European Union 2004, L(348):18-26.
3. Krech AB, Wurz A, Stemmer C, Feix G, Grasser KD: Structure of genes encoding chromosomal HMG1 proteins from maize. Gene 1999, 234(1):45-50.
4. Commission Regulation (EC) No 65/2004 of 14 January 2004 establishing a system for the development and assignment of unique identifiers for genetically modified organisms. Official Journal of the European Union 2004.
5. Corbisier P, Bhat S, Partis L, Xie VR, Emslie KR: Absolute quantification of genetically modified MON810 maize (Zea mays L.) by digital polymerase chain reaction. Analytical and bioanalytical chemistry 2010, 396(6):2143-2150.
6. Novick RP: Plasmid incompatibility. Microbiological reviews 1987, 51(4):381-395.
7. Sambrook J, Russell D: Molecular Cloning: A Laboratory Manual (Third Edition); 2001.
8. Held PG: Measure your purity: assessment of nucleic acid purity via UV absorbance. GIT Laboratory J 2008, 5-6/2008:9-11.
9. Event-specific Method for the Quantification of Maize 98140 Using Real-time PCR. In: EURL-GMFF: validated method maize 98140. European Commission, Commission, Joint Research Centre, Institute for Health and Consumer Protection (IHCP), Molecular Biology and Genomics Unit - European Union Reference Laboratory for GM Food and Feed (EURL-GMFF); 2011.
10. TaqMan® Universal PCR Master Mix. Available via http://docs.appliedbiosystems.com/pebiodocs/04304449.pdf. Applied Biosystems; 2010.
11. Caprioara-Buda M, Trapmann S, Gancberg D, Corbisier P, Merveillie A, Contreras Lopez MC, Emons H: Certification of Maize 98140 Reference Material ERM®-BF427c. ISBN 978-92-79-19279-1. EC certification report EUR 24718 EN. European Commission, Joint Research Centre, Institute for Reference Materials and Measurements; 2011.
12. Murray MG, Thompson WF: Rapid isolation of high molecular weight plant DNA. Nucl Acids Res 1980, 8(19):4321-4325.
13. Corbisier P, Broeders S, Charels D, Trapmann S, Vincent S, Emons H: Certification of plasmidic DNA containing MON 810 maize DNA fragments. ISBN 978-92-79-07139-3. EC certification report EUR 22948 EN. European Commission, Joint Research Centre, Institute for Reference Materials and Measurements; 2007.
14. Zhang Z, Schwartz S, Wagner L, Miller W: A greedy algorithm for aligning DNA sequences. J Comput Biol 2000, 7(1-2):203-214.
15. Definition of Minimum Performance Requirements for Analytical Methods of GMO Testing. Available via http://gmo-crl.jrc.ec.europa.eu/doc/Min_Perf_Requirements_Analytical_methods.pdf. European Network of GMO Laboratories (ENGL) Community Reference Laboratory (CRL); 2008.
16. Charels D, Broeders S, Corbisier P, Trapmann S, Schimmel H, Linsinger T, Emons H: Toward metrological traceability for DNA fragment ratios in GM quantification. 2. Systematic study of parameters influencing the quantitative determination of MON 810 corn by real-time PCR. Journal of agricultural and food chemistry 2007, 55(9):3258-3267.
17. International vocabulary of metrology - Basic and general concepts and associated terms (VIM). JCGM, 200:2008.
18. Corbisier P: Application Note 5. Use of Certified Reference Materials for the quantification of GMO in DNA copy number ratio (http://www.irmm.jrc.be/reference_materials_catalogue/user_support/erm_application_notes/application_note_5/Documents/application_note_5_english.pdf); 2007.

ANNEX 1: Primer and probe sequences used for the quantification of the 98140 event-specific real-time PCR.

	Sequence (5' to 3')	Reference
98140 primer F	5'-GTG TGT ATG TCT CTT TGC TTG GTC TT-3'	[9]
98140 primer R	5'-GAT TGT CGT TTC CCG CCT TC-3'	[9]
98140 probe	5'-(6-FAM)-CTC TAT CGA TCC CCC TCT TTG ATA GTT TAA ACT-(TAMRA)-3'	[9]
ZM1-F	5'-TTG GAC TAG AAA TCT CGT GCT GA-3'	[3]
ZM1-R	5'-GCT ACA TAG GGA GCC TTG TCC T-3'	[3]
ZM1 probe	5'-(6-FAM)-CAA TCC ACA CAA ACG CAC GCG TA-(TAMRA)-3'	[3]

ANNEX 2: Example for the preparation of calibration curves for the endogenous and transgenic targets.

An example for the preparation of calibration curves for the endogeneous and transgenic targets is given in Table 4. Each tube of ERM-AD427 calibrant is sufficient to prepare 10 calibration curves for each target (see also ERM Application Note 5 [18]).

Table 4: Preparation of calibration curves for the endogenous and transgenic targets

Starting concentration ~ cp/µL	**Resulting concentration** ~ cp/µL	**PCR target** [1]	**PCR target** [2]	**Dilution factor**	**DNA** [µL]	**Plasmid dilution buffer** [µL]
2 000 000	500 000			4	50	150
500 000	100 000	E		5	50	200
100 000	20 000	E		5	50	200
20 000	10 000		T	2	100	100
10 000	2 000	E	T	5	50	200
2 000	1 000	E		2	100	100
1 000	200	E	T	5	50	200
200	20		T	10	50	450
20	5		T	4	50	150

[1] "E" refers to the copy number concentrations of the pDNA calibrant that will be used in real-time PCR for the detection of the endogenous target (namely 100 000 cp/µL, 20 000 cp/µL, 2 000 cp/µL, 1 000 cp/µL and 200 cp/µL).

[2] "T" refers to the copy number concentrations of the pDNA calibrant that will be used in real-time PCR for the detection of the transgenic target (namely 10 000 cp/µL, 2 000 cp/µL, 200 cp/µL, 20 cp/µL and 5 cp/µL).

European Commission

EUR 24722 EN – Joint Research Centre – Institute for Reference Materials and Measurements
Title: Certification of Plasmid DNA containing 98140 Maize DNA Fragments - Certified Reference Material ERM®-AD427
Author(s): M. Caprioara-Buda, P. Corbisier, D. Gancberg, S. Broeders, S. Trapmann, S. Mazoua, G. Matayron, H. Emons
Luxembourg: Publications Office of the European Union
2011 –32 pp. – 21.0 x 29.7 cm
EUR – Scientific and Technical Research series – ISSN 1018-5593
ISBN 978-92-79-19277-7
doi:10.2787/38563

Abstract

This report describes the preparation, characterisation, stability and suitability of the certified reference material (CRM) ERM®-AD427, which contains a plasmid (pIRMM-0090) carrying a defined 2'-deoxyribonucleic acid (DNA) fragment specific for a genetic modification present in *Zea mays* L. line 98140, as well as a defined DNA fragment specific for the *Zea mays* taxon.

The CRM was processed in 2008 and certified in 2010 by the European Commission, Joint Research Centre, Institute for Reference Materials and Measurements (IRMM), Geel, BE. The CRM is available in plastic tubes containing 500 µL of the plasmid DNA in 1 mmol/L Tris, 0.01 mmol/L EDTA, pH 8.0 buffer (TE) and 1 ng/µL ColE1 plasmid used as background DNA. Each tube contains approximately 10^9 copies of the ERM-AD427 plasmid, which corresponds approximately to 3.3 ng of DNA.

The plasmid contains a 80 bp fragment specific for the maize 98140 and referred as the 5' insert-to-plant junction. Additionally, the plasmid contains a 79 bp fragment of the maize endogenous *high mobility group* (*hmg*) gene.

The certified values are the numbers of cloned DNA fragments for the 98140 and *hmg* PCR targets per plasmid.

The recommended minimum sample intake is 50 µL to perform a dilution series. A minimum sample intake of 5 µL was used per real-time Polymerase Chain Reaction (PCR) assay.

The intended use of this CRM is for the calibration of the event-specific method for the quantification of the 98140 event validated by the European Union Reference Laboratory for GM Food and Feed (EURL-GMFF), as described in the "Protocol for event-specific quantification of DP-Ø9814Ø-6 maize", accessible on the homepage of EURL-GMFF (http://gmo-crl.jrc.c.europa.eu/statusofdoss.htm)

The mission of the JRC is to provide customer-driven scientific and technical support for the conception, development, implementation and monitoring of EU policies. As a service of the European Commission, the JRC functions as a reference centre of science and technology for the Union. Close to the policy-making process, it serves the common interest of the Member States, while being independent of special interests, whether private or national.

JRC
EUROPEAN COMMISSION

LA- NA- 24722- EN- C

Publications Office

ISBN 978-92-79-19277-7